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The translation of the messenger for the poly(A)-binding protein-associated with translated mRNA is suppressed

A case of cytoplasmic repression in duck erythroblasts

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In vivo protein synthesis in duck erythroblasts was compared to in vitro translation of polyribosomal and free cytoplasmic mRNA. The in vivo study showed the absence of de novo synthesis of the M_r 73000 poly(A)-binding protein found associated with all polyribosomal mRNA. In vitro translation demonstrated that the mRNA for this protein is absent from the polyribosomal mRNA fraction but constitutes a medium frequency messenger among the repressed free mRNA. This result confirms the existence of a qualitative translational control in terminal differentiating duck erythroblasts leading eventually to the arrest of the protein synthesizing machinery.

Cytoplasmic repression *Poly(A)-binding protein* *mRNA translation* *Messenger suppression*
Protein synthesis *(Duck erythroblasts)*

1. INTRODUCTION

The presence, in the cytoplasm of eukaryotic cells, of untranslated mRNA maintained in the form of inactive free cytoplasmic mRNA–protein complexes, is now well established (see [1]). Evidence comes from studies on mRNA metabolism and translation during oogenesis and early embryogenesis [2–6], as well as in differentiated cells and tissues [7–12]. The biochemical basis for the difference in functional properties of translationally active (polyribosomal) and inactive mRNA protein complexes (mRNP), is however not clearly understood.

Previous studies from our laboratory, using duck erythroblasts, suggested a role of mRNP and mRNA associated proteins in the qualitative and quantitative regulation of protein synthesis contributing to the extraordinary specialisation of erythroid cells during terminal differentiation. In the cytoplasm of these cells, 200 different mRNAs are found in polyribosomes whereas 1200 other mRNA species are only detected in translationally inactive mRNP [12]. Among these mRNAs, we detected as a prominent species the mRNA coding for a basic M_r 73000 polypeptide, identified by two-dimensional gel electrophoresis as the major mRNP protein, the protein bound to the poly(A)

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Abbreviations: pre-mRNA, pre-messenger RNA; mRNA, messenger RNA; ScRNA, small cytoplasmic RNA; mRNP, complex messenger RNA–protein; EAT, Ehrlich ascites tumor; kDa, kilodalton

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sequence of polyribosomal mRNA [13,14]. This protein has been identified in numerous cell types and shows a high degree of evolutionary conservation [15]. Several roles have been suggested for the poly(A)-binding protein including a role in mRNA translation since, although polyadenylated, it is not associated with non-translated mRNA [16].

In view of: (1) the contribution of the cytoplasmic phase to the control of gene expression resulting in an increasing proportion of haemoglobin among the products of protein synthesis in the course of terminal differentiation; and (2) the potential role of the cytoplasmic poly(A)-binding protein in the regulation of mRNA translation; we investigated the synthesis of this particular protein in duck erythroblasts by in vivo protein radiolabelling. Products of in vivo protein synthesis were compared to the products of in vitro translation of polyribosomal and free cytoplasmic mRNA. The results of this comparison confirm the existence of a qualitative control of protein synthesis at the level of translation in duck erythroblasts as shown in [12]. Furthermore, they show that the M_r 73000 protein bound to the poly(A) segment of translated mRNA is not synthesized in duck erythroblasts and must therefore accumulate during previous stages of differentiation. These results are interpreted in the context of possible mechanisms leading to the restriction of protein synthesis paralleling the terminal specialisation of erythropoietic cells.

2. METHODS

2.1. *Incubation of cells and preparation of polyribosomes*

Immature erythroblasts were isolated from the blood of anemic ducks and resuspended in 4 vol. plasma as in [17]. The plasma was supplemented with [^{35}S]methionine (500 μCi/ml cells), 0.1 mM hemin and 20 mM Hepes buffer (pH 7.0). The cells were incubated for 3 h at 38°C (or 41°C) with a gentle rocking motion; 15 min prior to the end of incubation, emetine in 0.14 M NaCl was added to 0.2 mg/ml final conc. to prevent 'run-off' of ribosomes. After incubation, the cells were washed in a balanced salt medium, and lysed by controlled (2.5 min) hypotonic shock. The polyribosomes and free mRNP were isolated as in [18].

2.2. *Kinetics of incorporation of [^{35}S]methionine in vivo into total and cytoplasmic proteins*

2.2.1. Total synthesis of proteins

Cells (5 μl) were removed at different times, and the precipitable radioactivity/cell was determined. The number of cells in 5 μl aliquots were counted in the light microscope.

2.2.2. Radiolabelling of cytoplasmic proteins

Aliquots (100 μl) of cells were removed at indicated times, washed and lysed as above. The precipitable radioactivity of 10 μl post-mitochondrial lysate was determined. The radioactivity was normalized according to Hb concentration [19], for the specific activity the concentration of proteins was determined according to [20].

2.3. *Isolation of polyribosomal mRNP by oligo(dT)-cellulose*

Pellets of polyribosomes isolated from duck erythroblasts (section 2.1) were resuspended in 10 mM TEA–HCl (pH 7.4), 50 mM KCl, 5 mM β-mercaptoethanol and dissociated with EDTA prior to affinity chromatography on oligo(dT)-cellulose as detailed in [13]. Elution of bound material was by 50% formamide containing 10 mM TEA–HCl (pH 7.4), 10 mM EDTA (pH 7.4) and 50 mM NaCl.

2.4. *Preparation of polyribosomal and free poly(A)$^+$mRNA*

Polyribosomes and free mRNP were resuspended in 10 mM Tris–HCl (pH 7.4), 10 mM NaCl, 10 mM EDTA (pH 7.4), 0.5% SDS and digested with 200 μg/ml of proteinase K (1 h at 4°C). The RNA was extracted with hot (65°C) phenol at pH 8.0 as in [12], precipitated overnight with 2.5 vol. cold ethanol at −20°C. After centrifugation, RNA was dissolved in 10 mM Tris (pH 7.4), 10 mM NaCl. Poly(A)$^+$mRNA was prepared by chromatography at 18°C on an oligo(dT)-cellulose column.

2.5. *In vitro mRNA translation*

Template activity of mRNA was tested in vitro in two systems:

(A) A micrococcal nuclease-treated rabbit reticulocyte lysate [21]: the 20 μl reaction mixture containing 0.7 μg mRNA from polyribosomal

or free mRNP and 5 μCi [^{35}S]methionine was incubated 1 h at 30°C;

(B) Ehrlich ascites tumor cell-free system: the lysate prepared according to [32], was made RNA-dependent by micrococcal nuclease treatment [21]. The reaction mixture contained 25% lysate, 2 mM Mg-acetate, 150 mM K-acetate, 1 mM DTT, 1 mM ATP, 0.5 mM GTP, 40 μg of each amino acid except methionine, 50 μg/ml rat liver tRNA, 50 mM spermine, 500 μCi/ml [^{35}S]methionine, crude initiation factors from a reticulocyte lysate [22] at 0.3 mg/ml final conc. and mRNA at 35 μg/ml. Incubation was for 2 h at 30°C.

2.6. *Electrophoretic analysis of the in vivo and in vitro translation products*

One-dimensional and two-dimensional analyses of proteins were done as in [23,24] and radioactive proteins were detected using the method in [25].

3. RESULTS

3.1. *Analysis of in vivo synthesized proteins of duck erythroblasts*

Immature erythroid cells in the circulating blood of anemic ducks or chicken are normal diploid but non-proliferating cells, which synthesize pre-mRNA and mRNA [19] but neither DNA nor ribosomal RNA nor ribosomes; as a net result relatively few proteins are formed. We decided to investigate the pattern of proteins synthesized in duck erythroblasts by radiolabelling proteins in intact isolated cells. Erythroblasts were separated from white blood cells, reticulocytes and erythrocytes by several rounds of low speed centrifugation in complete synthetic medium (Gibco F13) and incubated for various periods of time in 4 vol. non-dialysed duck serum supplemented with haemin and [^{35}S]methionine (the use of synthetic media, devoid of the radiolabelled amino acid, is not possible since the cells generally develop rapid intra-cytoplasmic vacuolisation). In the presence of serum (see section 2), the incorporation of precursor amino acids into proteins increased regularly for a period exceeding 2 h (fig.1). The specific activity of proteins also increased linearly in function of time for 150 min, indicating no deprivation of the amino acid supply (fig.1). Therefore, 2 h incubation were chosen as the optimum time to qualitatively define the proteins synthesized in duck erythroblasts. Since in the cell, the time required for the complete synthesis of an average M_r (50000) protein is about 2 min [27] and the half life of mRNA is generally more than several hours [28,30], proteins labelled after 2 h may be considered as reflecting a steady state situation. In fact, analysis of proteins in cell samples taken after various times of labelling, ranging from 0.25–3 h, showed no major variation in the patterns of labelled proteins (not shown). No difference in labelled polypeptides was observed when the cells were incubated at 41°C, the body temperature of birds (not shown).

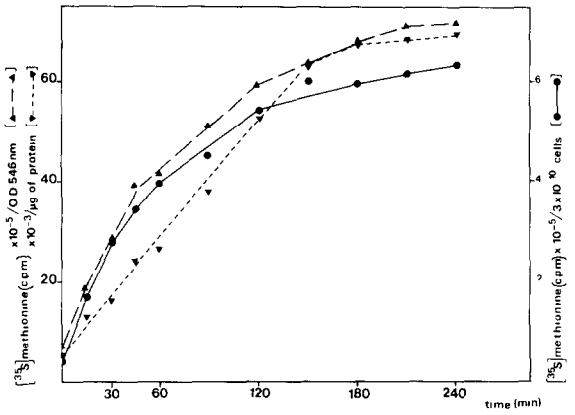

Fig.1. Kinetics of incorporation of [^{35}S]methionine into total and cytoplasmic proteins of duck erythroblasts. The experiment was done as in section 2: (●) total incorporation/cells; (▲) incorporation in cytoplasmic proteins/Hb; (▼) specific activity.

Fig.2 shows the two-dimensional electrophoretic pattern of cytoplasmic proteins from duck erythroblasts. Within the limits of this method (very basic polypeptides are not resolved on such a gel) most of the stained major cytoplasmic proteins (fig.2A), find a corresponding radioactive spot revealed by fluorography (fig.2B). This result indicates that most abundant cytoplasmic proteins are constantly synthesized in duck erythroblasts. However, except globin chains, only few erythrocyte specific proteins have been described thus far, and the study of the expression of cytoplasmic proteins specific of erythroid cell differentiation is still in progress [10,11].

A major protein escaping the previous conclusion is a basic 73 kDa polypeptide (⟶, fig.2)

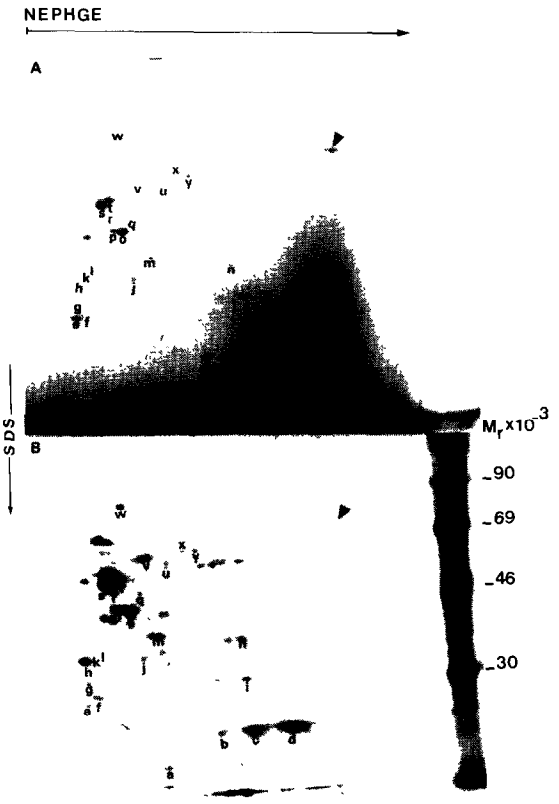

Fig.2. Two-dimensional analysis of a post-mitochondrial lysate from cells incubated with [^{35}S]methionine. After incubation of cells for 2 h in the presence of [^{35}S]methionine, 5 μl lysate containing about 4×10^6 cpm was solubilized by addition of 20 μl 9.5 M urea, 2% Nodinet P-40, 2% ampholines (pH 3.5–10), 5% 2-mercaptoethanol. The two-dimensional analysis used isotachophoresis in the first dimension [24], and in the second, electrophoresis in polyacrylamide–SDS gels [23]. After the second dimension, the gel was stained with Coomassie blue (A), and the labelled cytoplasmic proteins were detected by fluorography (B). Letters indicate the major proteins in constant synthesis, the arrow indicates the position of the poly(A)-binding protein.

which is always detected among stained polypeptides but has no counterpart among newly synthesized polypeptides. This polypeptide, easily separated from the bulk of acidic proteins has been identified by two-dimensional electrophoresis as the poly(A)-binding protein bound to the 3′-end of polyribosomal mRNA [13], and associated with the residual protein skeleton of erythroblast nuclei [29]. The absence of de novo synthesis of this protein in duck erythroblast confirms the data of in vitro translation of mRNA of polyribosomal and free mRNP; i.e., the mRNA coding for this protein is predominantly found in the translationally not expressed mRNP (cf. [14] and fig.4). Since a large free cytoplasmic pool of the 73 kDa poly(A)-binding protein exists (fig.2A; Maxwell, unpublished), there was the possibility that in erythropoietic cells, only that fraction of the poly(A)-binding protein associated with the new mRNA entering the cytoplasm was actively synthesized. We addressed this question directly by analysing the proteins of mRNP in polyribosomes

Fig.3. Analysis by polyacrylamide gel electrophoresis of polyribosomal mRNP proteins isolated by oligo(dT)-cellulose chromatography. Duck erythroblasts were incubated 3 h in the presence of [^{35}S]methionine at 38°C: (a) Coomassie blue stained polypeptides; (b) fluorography of polypeptides labelled in vivo. The arrows indicate the position of the 73 kDa poly(A)-binding protein.

of cells incubated for 2 h in the presence of [^{35}S]methionine. RNA labelling experiments had already shown that a large fraction of mRNA entering the cytoplasm associates with polyribosomes [17] and can be recovered as polyadenylated polyribosomal mRNP [14]. A crude preparation of mRNP was obtained by oligo(dT)-cellulose chromatography of EDTA-dissociated polyribosomes. The drawback of this quick mRNP isolation procedure is the large contamination of mRNA by ribosomal subunits (discussion in [13]) which, however, did not interfere in the present experiments; on the contrary, the ribosomal proteins provided an interesting control.

As observed with total cytosolic proteins (fig.2), no labelling of the 73 kDa protein bound to translated mRNA could be detected (fig.3). Some other polypeptides with M_r 50000–120000, previously identified as acidic mRNP proteins [13] are synthesized de novo contrary to the poly(A)-binding protein and the ribosomal proteins (M_r 15000–50000 [16]). The absence of synthesis of ribosomal proteins in duck erythroblasts contrasts with what occurs in dividing cells [19–31] but correlates with the absence of synthesis of ribosomal RNA or ribosomes reported in terminally differentiating

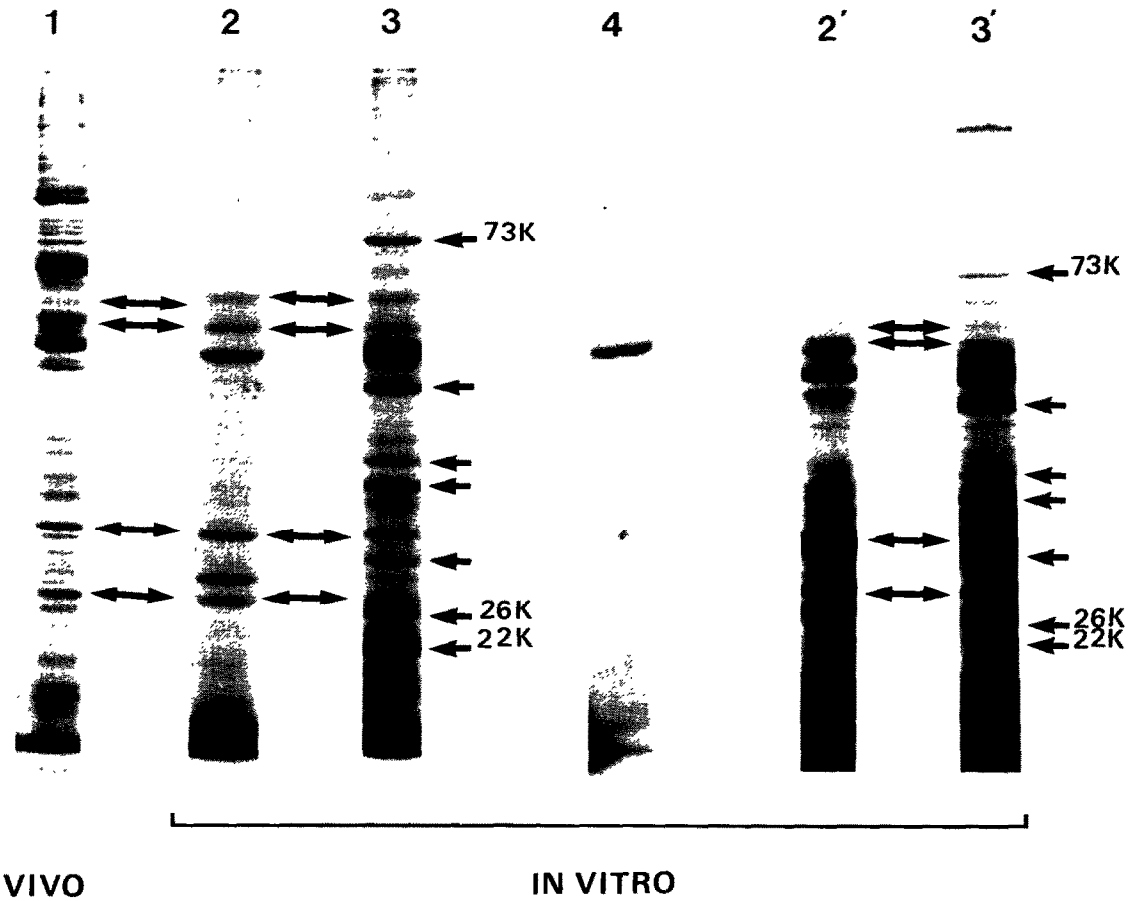

Fig.4. Comparison by polyacrylamide gel electrophoresis of cytoplasmic proteins labelled in vivo with the in vitro translation products of mRNA isolated from polyribosomal and free mRNP. In vivo and in vitro protein labelling was as in section 2: (a) in vivo labelled proteins; (2) and (2′) in vitro translation products directed by mRNA from polyribosomal mRNP: EAT cell free system (2) and rabbit reticulocyte lysate (2′); (3) and (3′) in vitro translation products directed by mRNA from free mRNP EAT cell free system (3) and rabbits reticulocyte lysate (3′); (4) endogeneous synthesis in the EAT cell free system without added mRNA (2′) and (3′) were analyzed on a separate gel).

erythropoietic cells [17]. This fact most probably represents a factor contributing to the low number of mRNA species translated in duck erythroblasts, compared to other cell types [12]: examination of the mRNA distribution in the duck erythroblast cytoplasm revealed that most of the 200 mRNA sequences detected in polyribosomes have their counterpart in the non-translated mRNP while about 1200 other mRNA species are uniquely detected in the free mRNP fraction [12].

3.2. *Comparison of in vivo protein synthesis with the products of polyribosomal and free mRNP templates in vitro*

Two different acellular protein synthesizing systems, the reticulocyte lysate and the Ehrlich ascites extract [21,32], were used for this comparison illustrated in fig.4. A good correlation between in vivo labelled proteins and in vitro translation products of polyribosomal mRNA was observed (⟶, fig.4). However, although subsaturating amounts of mRNA were used for this translation to minimize mRNA competition for initiation factors, large molecular weight polypeptides are in general under-represented, due to the high translational efficiency of globin mRNA. Nevertheless, it follows from this comparison that some major translation products of mRNA from free mRNP have no detectable counterpart among in vivo labelled proteins, or the translation products of polyribosomal mRNA. Among these, some clear examples are the 22 kDa and 26 kDa polypeptides first identified during the characterization of purified free cytoplasmic mRNA [16] and the 73 kDa polypeptide identified as the poly(A)-binding protein [14]. This comparison of the in vivo and in vitro synthesized polypeptides complements the original in vitro translation study [12] and reinforces our conclusion that a selective control of the expression of specific genes acts at the level of translation in duck erythroblasts.

4. DISCUSSION

In our search for factors involved in differential control of mRNA translation we concentrated our efforts, during the past years, on the characterization of proteins and ScRNA associated with translated and untranslated mRNA. The results of our and other laboratories led us to propose a role for the proteins associated with repressed mRNA in the selective control of mRNA translation [33]. Here, we concentrated our attention on the synthesis of the 73 kDa poly(A)-binding protein which is the major protein bound to polyribosomal mRNA but which was not found associated with non-translated mRNA [16,34].

The poly(A)-binding protein has been found in various cells and organisms; it is highly conserved during evolution [15]. Other laboratories have already investigated the kinetics of synthesis and turnover of this protein in differentiated and dividing cells. In Hela cells [35], de novo synthesis and appearance in the cytoplasm of the poly(A)-binding protein was linked to the polyadenylation of pre-mRNA in the nucleus and transport to the cytoplasm of newly synthesized mRNA [35]. A role for this protein in the transport of mRNA from the nucleus to the cytoplasm was proposed [35], in agreement with the association of this protein with the poly(A) segment of both cytoplasmic and nuclear polyadenylated RNA (see [1]). However, in [36] the question of the exchange of this protein between its free and mRNA bound forms in the cytoplasm of mouse L cells was addressed; it was shown that this exchange was not dependent upon mRNA transcription. In [36] experiments were conducted in the presence of high doses of actinomycin D and one cannot exclude possible effects of the absence of synthesis of mRNA on the metabolism and turnover of mRNA associated proteins. An investigation [36] of the transcriptional and translational control of protein synthesis after serum stimulation of quiescent 3T3 cells showed that the production of a limited set of proteins, among them a 72 kDa polypeptide, was considerably enhanced during that period. The presence of actinomycin D abolished, however, the increase of the 72 kDa polypeptide. Based on their respective migrations on two dimensional gels and their tryptic cleavage patterns (Thomas and Vincent, unpublished), the latter turned out to be the poly(A)-binding protein identical in the duck and mouse [15].

In duck erythroblasts, the situation observed in regard to the synthesis of the poly(A)-binding protein is therefore very different to that in dividing cells. The possible relation between the arrest of production of this mRNA-binding protein and the gradual extinction of protein synthesis paralleling

the terminal differentiation of erythropoietic cells has yet to be determined. The nuclease-treated reticulocyte lysate depleted of its pool of poly(A)-binding protein does not translate exogeneous mRNA; this suggests that the presence of this protein is necessary for translation (N. Standart, unpublished). Its decay in the absence of de novo synthesis might be the direct cause of the arrest of protein synthesis in the course of terminal differentiation of the red cell. In view of the selective control of gene expression at the level of translation, we observed in duck erythroblasts ([12,38] and here), this possibility represents an attractive working hypothesis. Indeed, one of the last decisive steps of erythroid differentiation might thus be controlled at a very late stage of the 'cascade of regulation' controlling gene expression in eukaryotic cells [38].

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